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Virology 350 (2006) 184–191
VIROLOGY
www.elsevier.com/locate/yviro

The BRO proteins of *Bombyx mori* nucleopolyhedrovirus are nucleocytoplasmic shuttling proteins that utilize the CRM1-mediated nuclear export pathway

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Received 6 December 2005; returned to author for revision 27 December 2005; accepted 3 January 2006
Available online 14 February 2006

Abstract

The BRO proteins of *Bombyx mori* nucleopolyhedrovirus (BmNPV) display a biphasic pattern of intracellular localization during infection. At early times, they reside in the nucleus but then show both cytoplasmic and nuclear localization as the infection proceeds. Therefore, we examined the possibility of nuclear export. Using inhibitors, we reveal that BmNPV BRO proteins shuttle between the nucleus and cytoplasm. Mutations on the leucine-rich region of BRO proteins resulted in nuclear accumulation of transiently expressed proteins, suggesting that this region functions as a CRM1-dependent nuclear export signal (NES). On the contrary, mutant BRO-D with an altered NES did not show nuclear accumulation in infected cells, although protein production seemed to be reduced. RT-PCR analysis showed that the lower level of protein production was due to a reduction in RNA synthesis. Taken together, our results suggest that BRO proteins are nucleocytoplasmic shuttling proteins that utilize the CRM1-mediated nuclear export pathway.

Keywords: Baculovirus; BmNPV; BRO; Nuclear export; CRM1

Introduction

Bombyx mori nucleopolyhedrovirus (BmNPV) is a member of the Baculoviridae, a diverse family of viruses with circular double-stranded (ds) DNA genomes that are pathogenic for invertebrates, particularly insects of the order Lepidoptera. BmNPV has a genome of 128 kb that potentially encodes 136 genes (Gomi et al., 1997). Among these ORFs, five genes (*bro-a*, *bro-b*, *bro-c*, *bro-d*, and *bro-e*) were found to belong to a unique baculovirus multigene family (*baculovirus repeated orf*; *bro*) (Gomi et al., 1997; Kang et al., 1999). Multiple members of the *bro* gene family also have been reported in the genomes of other baculoviruses (Ahrens et al., 1997; Chen et al., 2001; Hayakawa et al., 1999; Kuzio et al., 1999; Nakai et al., 2003; Pang et al., 2001). Furthermore, homologues of the *bro* gene have been identified in other insect dsDNA viruses. It was shown that *Amsacta moorei* entomopoxvirus (EPV) and *Melanoplus sanguinipes* EPV contain homologues that were referred to as ALI family (Afonso et al., 1999; Bawden et al., 2000). They have been also reported in iridovirus and ascovirus (Jacob et al., 2001; Bideshi et al., 2003). These findings suggest that *bro* genes may be widespread among insect dsDNA viruses. Although the conservation among the dsDNA viruses suggests that *bro* genes serve an important role in infection, the function of this gene family remains unclear. It has been previously reported that all *bro* genes of BmNPV are actively transcribed as delayed early genes since *bro* genes require IE1 for their expression, and that BRO proteins are produced at high levels between 8 and 14 h postinfection (p.i.) (Kang et al., 1999). It has been also reported that one of the BmNPV *bro* genes (*bro-d*) is essential for viral infection, and that *bro-a* and *bro-c* may functionally complement each other (Kang et al., 1999). In addition, Zemskov et al. (2000) have shown that BmNPV BRO proteins have nucleic acid binding activity and are involved in nucleosome organization in infected cells.

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0042-6822/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.01.008

Eukaryotic cells possess a double nuclear membrane containing nuclear pores that regulate bidirectional transport of proteins and RNAs between the nucleus and the cytoplasm. The nuclear export of proteins and RNA is a signal-dependent process mediated by soluble receptors called exportins. The best characterized nuclear export signal (NES) was first described in the human immunodeficiency virus Rev protein (Fischer et al., 1995). NESs are generally short stretches of amino acids characterized by multiple hydrophobic residues with a conserved motif (Φ-X_{2-3}-Φ-X_{2-3}-Φ-X-Φ, where Φ indicates hydrophobic residues such as leucine, isoleucine, methionine, valine, or phenylalanine and X is any amino acid) that interacts with the export receptor (Kutay and Güttinger, 2005). The exportin CRM1 recognizes NES on cargo molecules targeted for export (Fornerod et al., 1997; Fukuda et al., 1997). As a specific inhibitor of CRM1 function, the cytotoxin Leptomycin B (LMB) provides a useful reagent for studying nuclear export in cells. LMB inhibits CRM1-mediated nuclear export of a range of proteins and RNAs by binding to the central domain of CRM1 to disrupt its interaction with the NES (Fornerod et al., 1997; Kudo et al., 1999; Nishi et al., 1994).

Baculovirus replication occurs in the host cell nucleus, consequently, the newly synthesized viral mRNAs in the nucleus must be exported to the cytoplasm, while some viral proteins produced in the cytoplasm are of necessity imported into the nucleus. However, our understanding of the nucleocytoplasmic transport of baculoviral products remains limited. At the subcellular level, BRO proteins of BmNPV are nuclear proteins during the early stage of infection such as 4 hpi but distribute to both the nucleus and the cytoplasm as the infection proceeds (Kang et al., 1999). This double localization suggests the possibility that BRO proteins undergo nuclear export. In this report, we examined this possibility and show that BRO proteins are indeed nucleocytoplasmic shuttling proteins that utilize the CRM1 mediated nuclear export pathway.

Results

BmNPV BRO proteins shuttle between the nucleus and the cytoplasm

We have previously reported that BmNPV BRO proteins distributed to the nucleus at 4 hpi and then localized both in the nucleus and the cytoplasm as the infection proceeds (Kang et al., 1999). Since this double localization suggested the possibility for nuclear export, we treated BmN cells with Leptomycin B (LMB), an inhibitor for CRM1-mediated nuclear export to test whether BRO proteins are exported by this general export receptor (Fornerod et al., 1997). If CRM1 is involved in BRO export, treatment with LMB should restrain the protein to the nucleus. BmN cells infected with BmNPV were treated with 10 nM LMB at 1 hpi and then analyzed by immunofluorescence staining 5 h later (6 hpi). In untreated cells, BRO localized both to the nucleus and the cytoplasm (Fig. 1, panels a and c). In contrast, cells treated with LMB showed a dramatic change in BRO distribution,

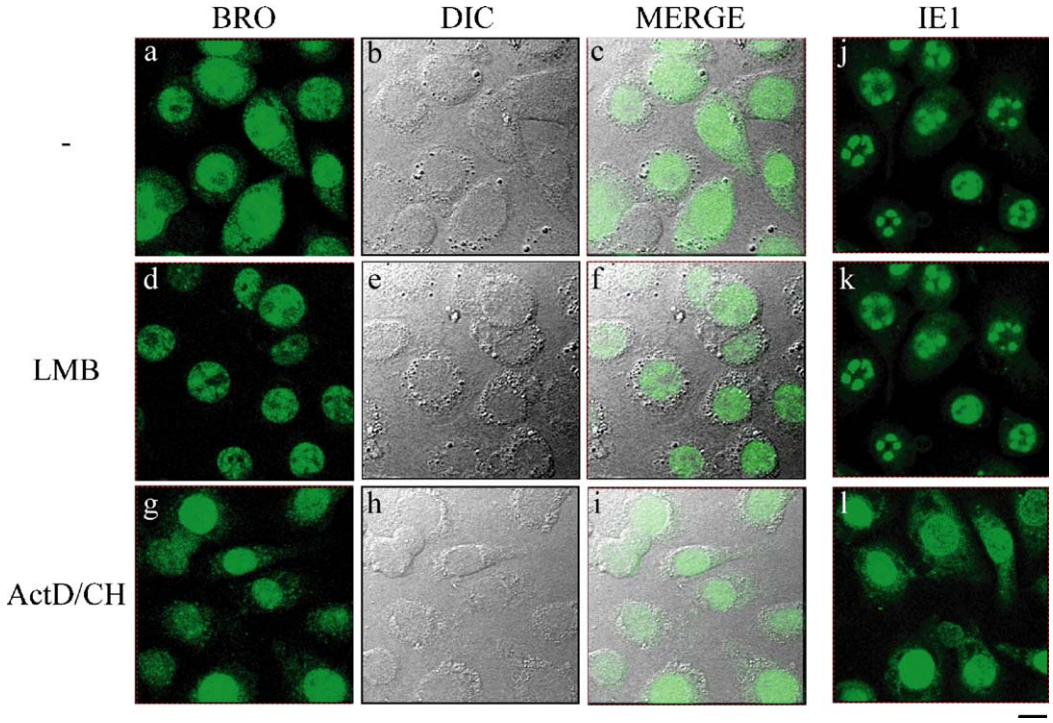

Fig. 1. Intracellular localization of BmNPV BRO and IE1 in the presence of inhibitors. BmN cells infected with BmNPV were incubated in the absence of any inhibitor (a–c, j) or presence of LMB (d–f, k) or ActD and CH (g–i, l). Panels a, d, g in the left show BRO immunofluorescence images using anti-BRO, and the panels b, e, h are differential interface contrast images of the same fields as the left ones. These were merged in the panels c, f, i. Panels j–l show IE1 immunofluorescence images using anti-IE1. The scale bar is 10 μm.